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GROWTH OF VEE AND EEE VIRUSES IN CHICK FIBROBLAST CULTURES AT HIGH TEMPERATURES

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ABSTRACT

The work reported here concerns the study of growth characteristics of Venezuelan equine encephalitis (VEE) and eastern equine encephalitis (EEE) viruses propagated in CF cells at high temperatures. VEE virus was able to form plaques at 44 C; EEE virus formed plaques to 46 C. Uninfected CF monolayers could tolerate a maximum temperature of 46 C, but were killed at about 47 C.

Maximal growth rates and virus yields in liquid overlay cultures were observed to 40 C for VEE virus. Marked inhibition of this virus occurred at temperatures greater than 40 C. No increase in virus titer could be detected at 44 C; at 46 C or greater virus titers dropped rapidly. The growth response of EEE was maximal to 44 C and substantial viral growth took place at 47 C. Further studies indicated that if CF monolayers infected with VEE virus were transferred from 37 C to 44 C at different stages during the viral growth cycle, virus replication was halted almost immediately. In another experiment in which virus-infected monolayers were transferred from 44 C after various periods of time to 37 C, virus replication was initiated within four hours after transfer.

These data indicate that the differences in the maximum growth temperatures for VEE and EEE viruses appear to be due to the existence of a virus-induced, temperature-sensitive step in VEE virus synthesis.

I. INTRODUCTION

The unusual capacity of chick fibroblast (CF) cells to tolerate temperatures to 46 C afforded the opportunity to study the growth response of animal viruses at elevated temperatures. The work reported here concerns the study of growth characteristics of Venezuelan equine encephalitis (VEE) and eastern equine encephalitis (EEE) viruses propagated in CF cells at high temperatures.

II. MATERIALS AND METHODS

The Trinidad strain of VEE virus and the Louisiana strain of EEE virus were used in these studies.

CF monolayers were prepared from minced trypsinized ten-day-old chick embryos. Twenty million to 30 million cells, contained in 5 ml of growth medium, were added to 60-mm petri dishes. The growth medium consisted of lactalbumin hydrolyzate, yeast extract, calf serum, and salts and will be referred to as lactalbumin hydrolyzate medium in this report. Confluent monolayers were obtained after 24 hours at 37 C in an incubator supplied with a mixture of 5% carbon dioxide in air.

Temperature studies were performed with a temperature gradient plate described by Brown* or in an incubator set at 44 C. A photograph of the temperature gradient plate is shown in Figure 1. The plate is an elongated, shallow metal box with the top and bottom hinged in back so that it opens like a sandwich grill. When the box is closed a chamber is formed that can hold four rows of 10- to 60-mm plates. The top and bottom of the chamber are one-fourth-inch aluminum plates, each heated at one end by a strip heating element controlled by a thermostat. Heat applied to one end of each plate dissipates toward the opposite end to provide a temperature gradient. A humidified mixture of 5% carbon dioxide in air was introduced into the chamber at four locations and vented at two other points.

In those studies involving plaque formation, the CF monolayers were washed twice with phosphate-buffered saline (PBS) and then infected with appropriate dilutions of VEE or EEE virus prepared in beef heart infusion broth. Virus was adsorbed for 30 minutes and the cultures were overlayed with lactalbumin hydrolyzate medium containing agar. Cultures were incubated at 37 C for two days. To determine cell viability and to detect plaques, the monolayers were then stained by applying a layer of agar containing 1:10,000 neutral red.

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* Brown, Arthur. 1963. Differences in maximum and minimum plaque-forming temperatures among selected group A arboviruses. Virology 21:362-372.

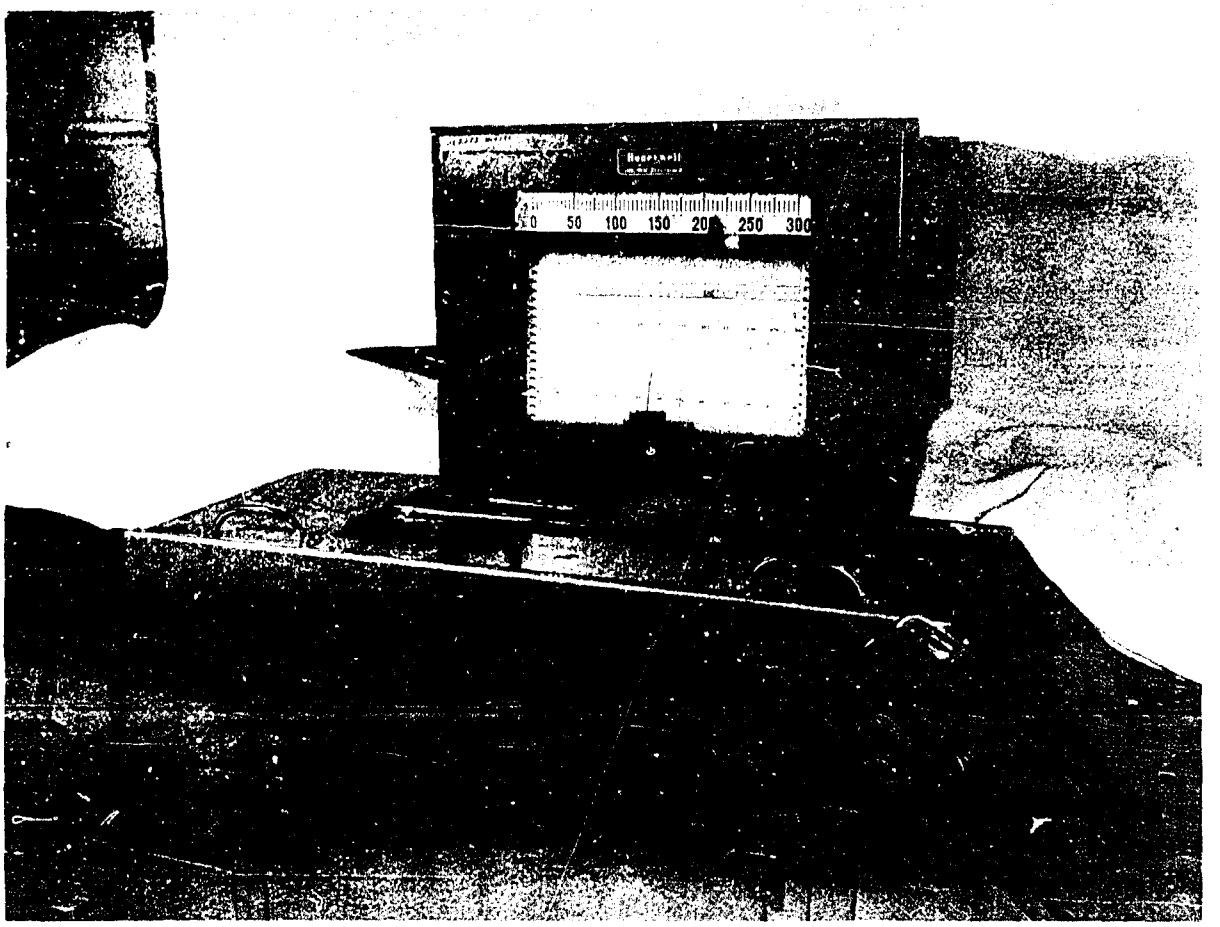

Figure 1. Photograph of Temperature Gradient Plate.
(FD Neg C-6971)

III. RESULTS

The first experiment was designed to determine the ability of VEE and EEE viruses to form plaques at elevated temperatures. CF monolayers were infected with each virus and then placed on the temperature gradient plate where the temperature range was adjusted between 37 C and 52 C.

CF monolayers were killed by incubation temperatures of 47 C or higher, as indicated by the loss of the ability to take up neutral red dye (Table 1). VEE virus was able to form plaques to a maximum temperature of 44 C; EEE virus was able to form plaques to 46 C under the same conditions.

TABLE 1. PLAQUE FORMATION BY VEE AND EEE VIRUS ON CHICK FIBROBLAST MONOLAYERS AT ELEVATED TEMPERATURES

Temperature, C	VEE	EEE
37	+[a]	+
40	+	+
42	+	+
44	+	+
46	0[b]	+
47	MD[c]	MD

a. + = plaques present, monolayers viable.
b. 0 = no plaques observed, monolayers viable.
c. MD = monolayers dead, based on inability to take up neutral red dye.

The capacity of VEE virus to form plaques at 44 C but not at 46 C is a sharply defined characteristic and can be used to separate a relatively small EEE virus population from a much larger number of VEE virus units simply by incubation at 46 C, as shown in Table 2. Data in the first column of the table were obtained from a 0.1-ml volume of a 10^{-4} dilution of VEE virus that contained approximately 10^4 plaque-forming units and was placed on each CF monolayer and then incubated in the temperature

gradient plate. Confluent plaques were formed to 43 C; at 46 C no plaques were observed, but monolayers appeared viable. In the second column when a 10^{-7} dilution of EEE virus was plated, discrete and countable plaques were obtained up to 46 C. The third column of this table shows the results obtained when the two dilutions of VEE and EEE virus, which contained excessive VEE virus, were mixed and used to infect CF monolayers. Confluent plaques were observed to 43 C but at 46 C relatively few plaques were obtained and these were the size of EEE and not of VEE plaques. When the same virus mixture was used to infect the monolayers and then anti-EEE serum was added to the overlay medium, confluent plaques were again observed to 43 C but no plaques were observed at 46 C. These data indicate that the plaques that appeared at 46 C from the VEE-EEE mixture were actually those of EEE virus.

TABLE 2. SEPARATION OF EEE VIRUS FROM VEE VIRUS AT ELEVATED TEMPERATURES

Temperature, C	VEE, 10^{-4} dilution[a]	EEE, 10^{-7} dilution[b]	VEE 10^{-4} dil + EEE 10^{-7} dil	VEE 10^{-4} dil + EEE 10^{-7} dil + Anti-EEE Serum[c]
37	Confluent plaques	96	Confluent plaques	Confluent plaques
41	Confluent plaques	96	Confluent plaques	Confluent plaques
43	Confluent plaques	79	Confluent plaques	Confluent plaques
46	0[d]	59	47	0
47	MD[e]	MD	MD	MD

a. 10^{-4} dilution VEE, approximately 10,000 plaque forming units per plate.
b. Number of plaques/plate.
c. Anti-EEE serum added to nutrient agar overlay.
d. 0 = no plaques observed, monolayers viable.
e. MD = monolayers dead, based on inability to take up neutral red dye.

These data further indicate that both viruses can form plaques in CF cells at unusually high temperatures; however, CF cells that are able to form EEE virus plaques at 46 C cannot form VEE virus plaques. It appears that a temperature-sensitive step is involved in the synthesis of VEE virus, but not of EEE virus. It seems likely that the sensitive step is induced by the virus and therefore reflects a genetic property of the virus.

In an effort to learn more about the temperature-sensitive mechanism involved in the synthesis of VEE virus, the growth responses of both VEE and EEE viruses under a liquid overlay medium were compared at different growth temperatures. Twenty-four-hour CF monolayers were infected at a multiplicity of 10. The virus was allowed to adsorb on the medium for 30 minutes. The infected monolayers were washed twice with 3 ml PBS and then overlayed with 5 ml lactalbumin hydrolyzate medium containing 10% calf serum. The plates were placed on the temperature gradient plate and 0.2-ml samples were withdrawn every two hours and assayed for virus titer. The cultures were not removed from the temperature gradient plate during the sampling procedure.

The growth of VEE virus at different temperatures is shown in Figure 2. Maximal growth rates and virus yield were observed at temperatures up to 40 C. Marked inhibition of VEE virus growth was noted at temperatures greater than 40 C. No increase in virus titer occurred at 44 C. At 46 C and greater, virus titers dropped rapidly.

The growth of EEE virus at various temperatures is shown in Figure 3. Maximal growth was obtained to 44 C and substantial virus growth was still observed at 47 C; thereafter virus titers dropped. At 30 hours after infection a detectable level of EEE virus was still present at 50 C, suggesting that some virus growth occurred even at this temperature.

The difference in the growth response of the two viruses to high temperatures does not appear to be related to their heat stability, since VEE virus held at 50 C in a cell-free medium was far more heat-stable than EEE virus. Thus, the difference in the maximal growth temperature of these two viruses appears to be due to differences in their ability to multiply in the host cell.

The data presented to this point suggest that a virus-induced enzyme system that is temperature-sensitive above 40 C is involved in the synthesis of VEE virus. The following studies were performed at the critical temperature of 44 C to determine the nature of the system involved.

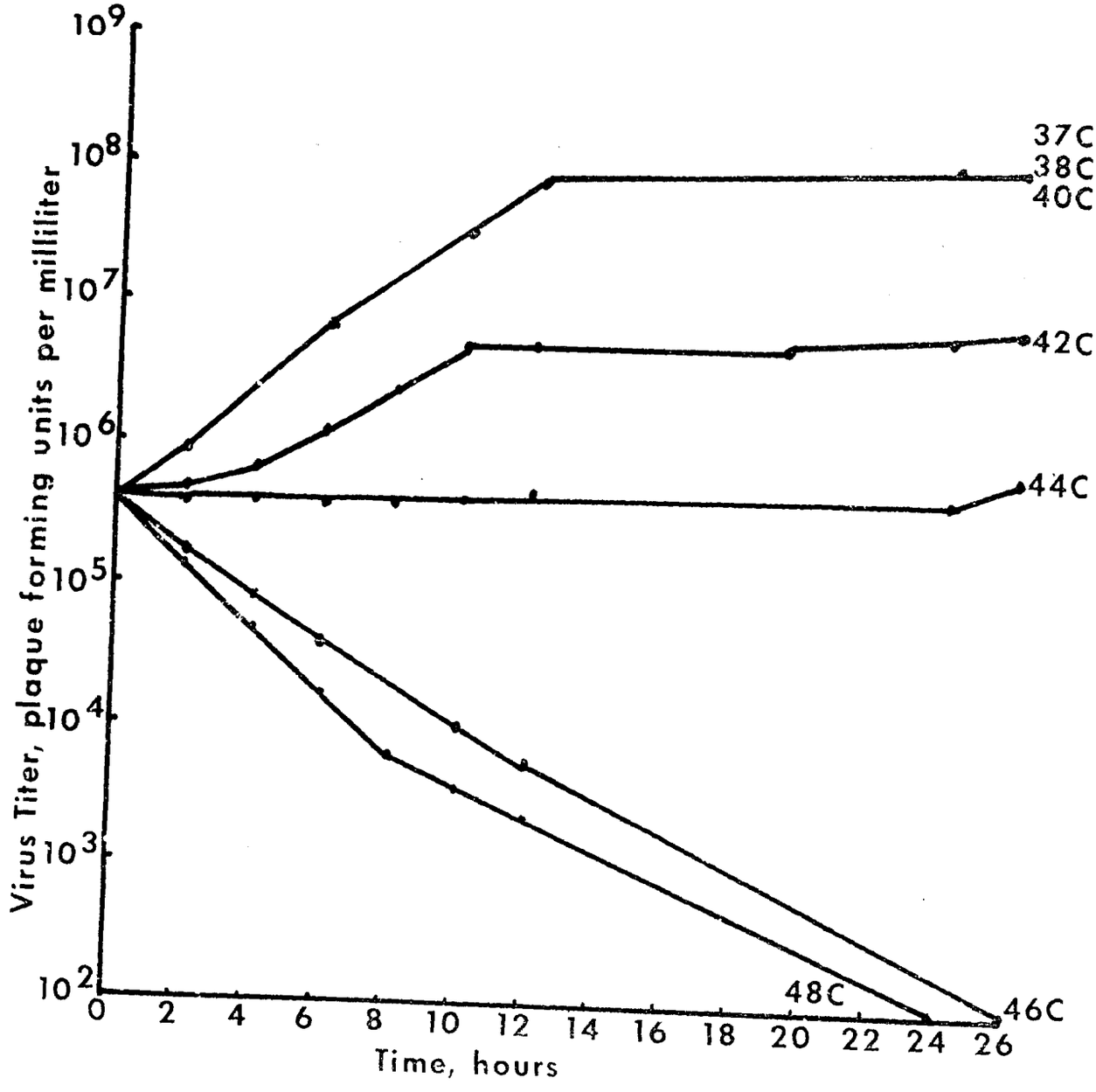

Figure 2. Growth Response of VEE Virus at Different Temperatures.

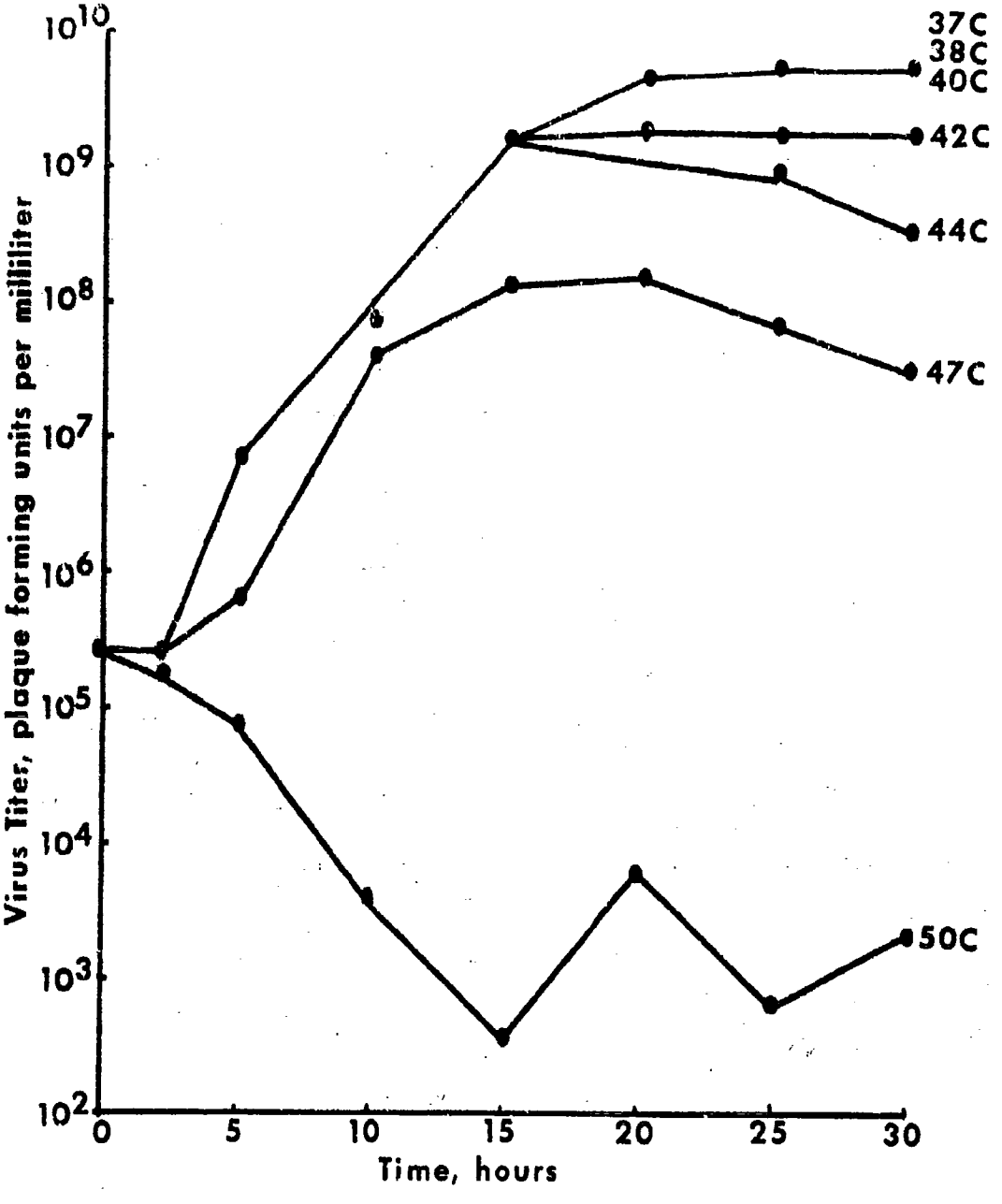

Figure 3. Growth Response of EEE Virus at Different Temperatures.

A large number of CF monolayers infected with VEE virus were prepared at the same time. A control group of plates was placed in a 44 C incubator and the remainder were incubated at 37 C. At hourly intervals one group of plates was transferred from the 37 C to 44 C incubator, and at the same time one plate from each temperature group was assayed for virus titer. With this system it was possible to determine the effect on the growth cycle of transferring cultures from 37 C to 44 C at different but sequential stages after infection. The results of a typical experiment are shown in Figure 4.

As cultures were transferred from 37 C to 44 C, virus replication was halted almost immediately. If the plates were transferred before two hours the growth curve followed that of the 44 C control culture. Transferring the cultures after two hours resulted in an apparent inhibition of virus replication so that the virus titer remained at approximately the same level that the cultures had at time of transfer.

The reverse type of experiment, the effect of transferring VEE virus-infected CF cultures from 44 C to 37 C, is shown in Figure 5. The procedure used for sampling and transferring cultures was the same as that just described except that the infected monolayers were placed initially at 44 C. Virus growth was initiated within four hours after transferring the cultures to the 37 C incubator. If the cultures are incubated at 37 C long enough, the virus titer will eventually reach that of the 37 C control culture. A similar experiment was performed in which the cultures were transferred from 44 C to 37 C at 18 hours or later after infection and again virus growth was initiated even after 28 hours at 44 C.

These data indicate that, although the virus-induced system appears to be inhibited by temperatures of 44 C, it does not seem to be irreversibly damaged because transferring the cultures back to 37 C results again in virus synthesis.

In summary, the data indicate that CF cells remain viable and permit VEE virus replication up to 44 C and EEE virus synthesis up to about 46 to 47 C. The difference in the maximum growth temperatures for these viruses appears to be due to the existence of a virus-induced temperature-sensitive step in VEE virus synthesis. VEE virus replication can be halted at any point during its growth cycle by transferring cultures to 44 C, and virus growth initiated by transferring the cultures back to 37 C, even after 18 hours at 44 C.

The temperature-sensitive step in VEE virus synthesis is as yet unknown; however, additional studies are underway to determine which stage of virus synthesis within CF cells is affected by high growth temperatures.

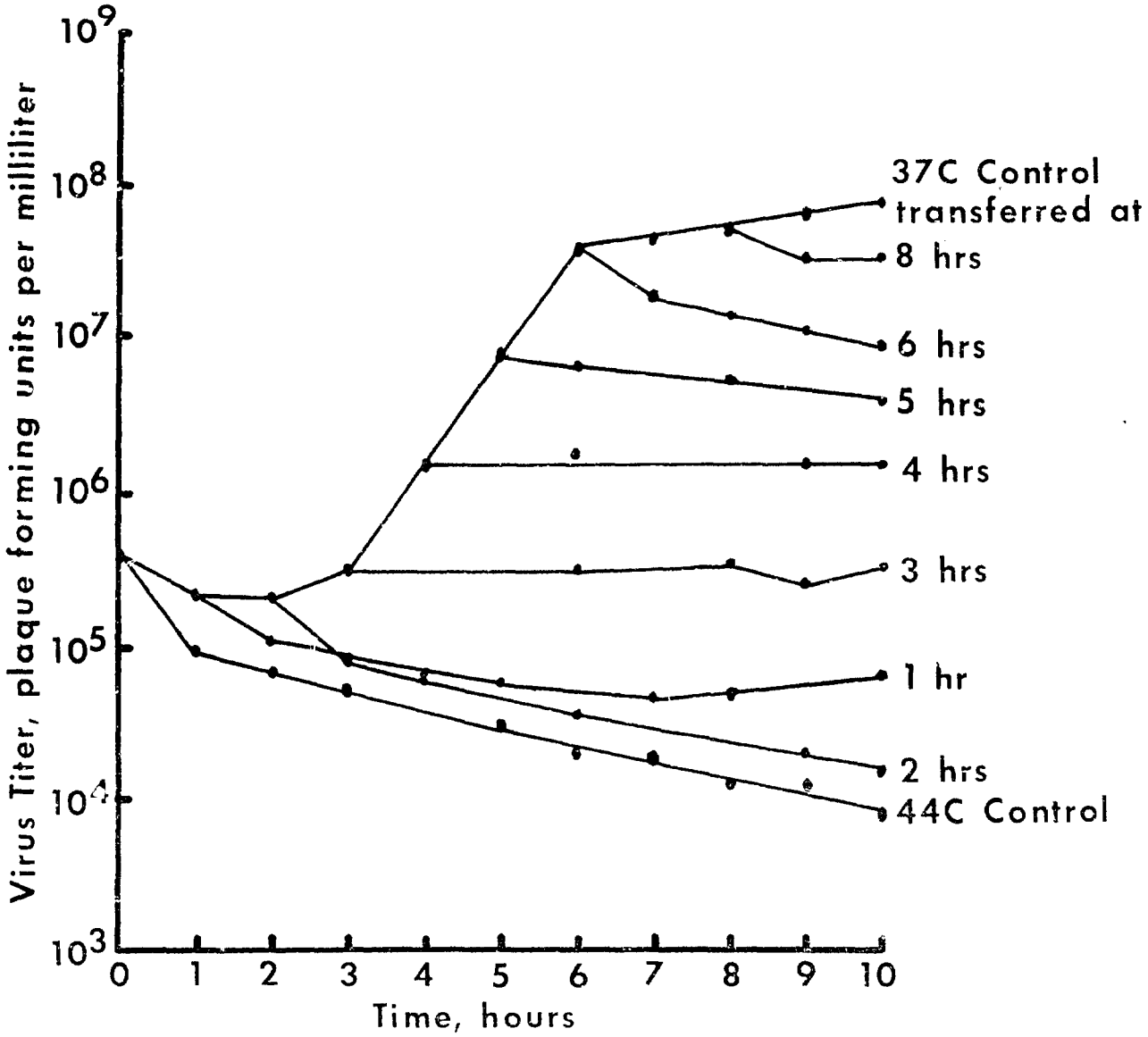

Figure 4. The Effect of Transferring Plates from 37 C to 44 C on Growth of VEE Virus.

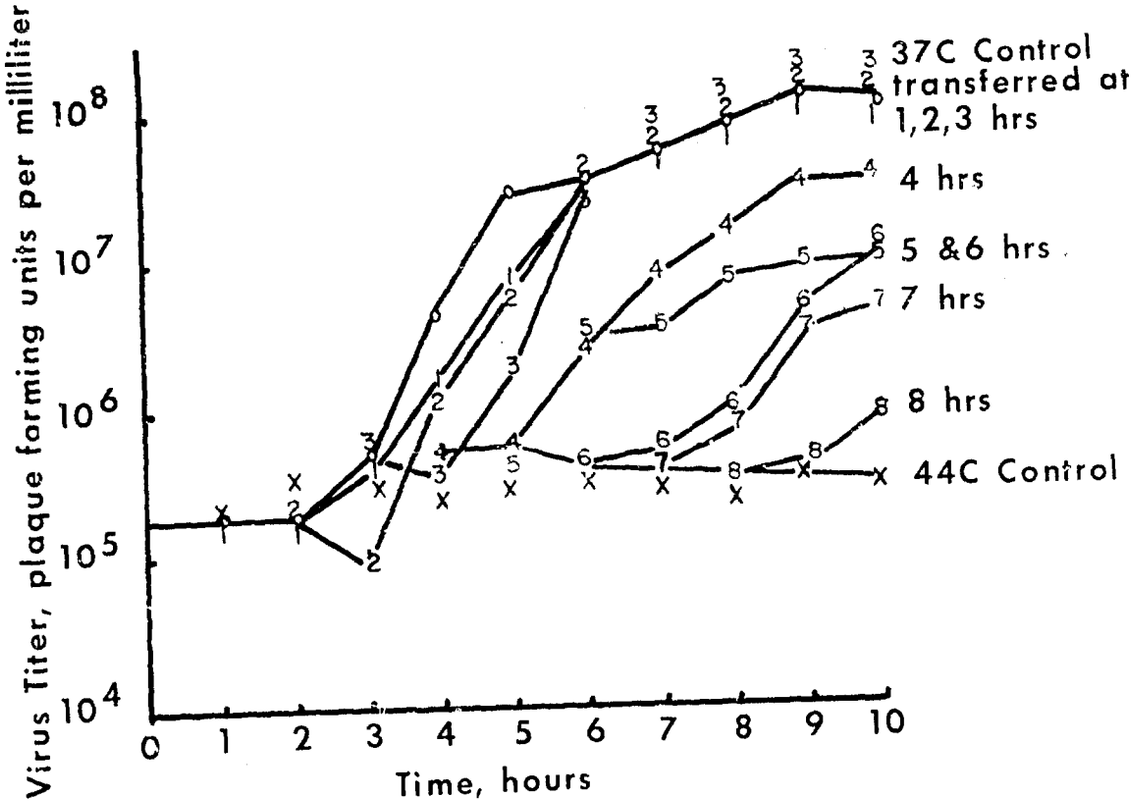

Figure 5. The Effect of Transferring Plates from 44 C to 37 C on Growth of VEE Virus.